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Serologically defined *Rhipicephalus* (*Boophilus*) *microplus* larval antigens in BmLF3, a partially pure Sephacryl S-300 fraction of crude larval proteins☆

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Abstract

This report is designed to provide additional information regarding larval soluble proteins toward the planned development of a comprehensive database of *Rhipicephalus (Boophilus) microplus* proteins that elicit a humoral immune response in cattle as a result of natural ectoparasite infestation. Larval proteins of *R. microplus* are complex and the protein profile is not dominated by any major proteins. This report focuses upon an S-300 Sephacryl (molecular sieve) column fraction, fraction 3 (BmLF3). With the use of SDS-PAGE (without-2ME) and Western blotting with a composite pool of pre- and post-*R. microplus* larval infestation antiserum BmLF3 was found to contain 7 apparent common ixodid major antigens (207.3, 171.9, 98.0, 86.5, 65.7, 58.9, and 38.0 kDa), those potentially shared with other ixodid species, and 2 apparent *R. microplus* specific antigens evidenced by low-level antibody binding in crude BmLF3 (149.4 kDa) and HPLC peak 8 of BmLF3 (116.0 kDa). In addition, BmLF3 contains potent inhibitors of trypsin activity. However, these inhibitors of trypsin did not appear to elicit host antibodies as a result of natural ectoparasite exposure, as defined by Western blotting of reduced and denatured trypsin binding proteins purified by affinity chromatography.
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Keywords: *Rhipicephalus (Boophilus) microplus*; Antigen; Natural exposure; Serological

1. Introduction

Rhipicephalus (*Boophilus*) *microplus* (Say), the southern cattle tick, vectors the causative haemoprotozoan agents of bovine babesiosis *Babesia bovis* and *Babesia bigemina.* As a result, *R. microplus* is a significant veterinary ectoparasite that limits livestock production in many parts of the world. Control of this vector is achieved primarily through the use of acaricides (Willadsen, 1997; Pruett, 1999). However, alternative and sustainable control technologies are increasingly desired. Donald (1994) suggested that the most sustainable method of control would be from natural or vaccine induced host resistance. Producers

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have exploited herd immunity in tropical climates by the selection of the naturally parasite resistant *Bos indicus* breeding stock (Morrison, 1989; de Castro and Newson, 1993). Natural immunity to *R. microplus*, acquired through repeated exposure, has been described as a partial and stable immunity (Wagland, 1975), characterized by immediate-type hypersensitivity, and is most effective against immature life-stages (Willadsen et al., 1978).

If natural resistance elicited by natural ectoparasite exposure can be made more effective, then herd immunity can potentially be maintained as a result of low-level natural ectoparasite exposure negating the need for booster immunization as with vaccines based upon concealed antigens (Pruett, 2002). However, tick proteins that elicit these natural immune responses are not well characterized (Brossard et al., 1991). In a recent report, we identified bovine serologically defined *R. microplus* larval antigens in a partially pure S-300 Sephacryl protein fraction, BmLF2 with antiserum from a single calf (Pruett et al., 2006). This study extends that database as we report on the identification of bovine serologically defined antigens in Sephacryl S-300 protein fraction BmLF2 and fraction 3, BmLF3, an additional partially purified fraction of crude *R. microplus* larval proteins with antibodies in pooled antiserum collected pre- and post-*R. microplus* larval infestation.

2. Materials and methods

2.1. Tick strains, tick extracts and protein determination

The Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX, maintains *R. microplus* ticks in quarantine. An acaricide susceptible control strain, the Munoz strain F_{21}, was used in this study for infestation and extraction of larval proteins. Soluble larval proteins were prepared from 5 g of 10–14-day-old larvae by homogenization in 10 mM sodium phosphate buffered saline, pH 7.2 (10 ml/g of tick larvae), as described previously in detail (Pruett et al., 2006).

2.2. Infestation of cattle for antibody production

Weaned Hereford calves, of approximately six months of age and presumably maintained on open pasture, with the potential for ixodid tick exposure, ($n = 8$; calf identification numbers 512, 515, 518, 522 for larval exposure, and calf identification numbers 513, 514, 516, 517 for complete life-cycle exposure) were purchased from the Gillespie Livestock Co. Inc., Fredericksburg, TX. The calves were in-processed at the Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL), Kerrville, TX where they were vaccinated and dewormed (SafeGuard, fenbendazole paste 10%, Hoescht-Roussel Agrivet, Sommerville, NJ) as previously described in detail (Pruett et al., 2006). They were then transported to the CFTRL for infestation with *R. microplus*. As stated above 4 calves received repeated infestations with only larval exposure. The detailed procedure for infestation of calves with larval *R. microplus* has been reported (Pruett et al., 2006). Briefly, calves were infested with larvae for six consecutive infestations with 0.5 g of larvae. To obtain larval life-stage specific antiserum, infestations were terminated 4 days following infestation by dipping the calves in 0.3% coumaphos before larval molt to the nymphal life-stage. Serum was collected from blood samples obtained before and after each exposure. Those calves exposed to the complete life-cycle (larvae, nymphs, and adults) of *R. microplus* were stanchioned and exposed to 1 g of larvae, free released on the calf. Blood samples were obtained on day 28 of infestation and the calves dipped (0.3% coumaphos) and released to pasture. Seven days later another blood sample was collected. The calves' convalescence continued for an additional 2 weeks on pasture, at which time they were bled and exposed to another infestation with *R. microplus*, as described above. This complete life-cycle infestation was repeated 3 times.

Composite pooled antisera used in Western blot experiments with crude BmLF2, BmLF3, and HPLC fractionated BmLF3 proteins, included two pools from larvae exposed calves, a sample pooled prior to the initial *R. microplus* exposure (pre-infestation), and a pool prepared from sera drawn 21 days following the fifth larval exposure (post-infestation). A third pool of serum was prepared from serum collected from calves 28 days following their third exposure to the complete life-cycle of *R. microplus*. Calf 522, a larvae only exposed calf, had the highest anti-larval antibody titer. Serum from this calf, collected prior to *R. microplus* exposure and 21 days following the fifth larval exposure was used in the Western blot experiment of the trypsin binding proteins in BmLF3 filtrate, along with the pooled serum from calves infested with the complete life-cycle stages of *R. microplus*.

2.3. Partial purification of fraction 3 (BmLF3) with open-column chromatography

Crude larval proteins were concentrated to 15 ml prior to chromatography with an Amicon ultrafiltration

device (Millipore Corp., Bedford, MA; YM 10 membrane, 10,000 molecular weight cutoff). BmLF3 fraction 3 is represented by those larval proteins in tubes 31–43 (10 ml/tube) of a Sephacryl S-300 gel filtration (Amersham Biosciences, Piscataway, NJ) of concentrated crude *R. microplus* larval proteins, as previously described (Pruett et al., 2006). Tubes 31–43 from the S-300 fractionation were pooled and concentrated to 15 ml as stated above. Both the concentrate and the filtrate were stored at −20 °C.

2.4. High-pressure liquid chromatography (HPLC) separation of BmLF3 proteins

BmLF3 proteins were further separated by reversed-phase HPLC with a Waters Xterra RP_{18}, 5 μM, 4.6 mm × 150 mm column (Waters Corp., Milford, MA). Proteins were eluted using a linear gradient from 0.1% trifluoroacetic acid (TFA), buffer A to 100% acetonitrile containing 0.1% TFA, buffer B over 67 min. The gradient was controlled by a Waters model 680 automated gradient controller (Waters Corp., Milford, MA). BmLF3 proteins were separated into 10 peaks and concentrated to dryness with a Savant Speed Vac SC110 (Savant Instrument Inc., Farmingdale, NY). Proteins were rehydrated in 50 mM sodium phosphate buffer, pH 7.5, with and without 0.1% sodium dodecyl sulfate (SDS).

2.5. SDS-PAGE and Western blotting

Fractionated proteins were resolved on 4–12% Bis–Tris, NuPAGE® gradient polyacrylamide gels (Invitrogen, precast gels, Carlsbad, CA) in a NuPAGE® MES (morpholineethanesulfonic acid) SDS running buffer according to the manufacturers instructions. Samples were prepared in SDS with and without 5% 2-mercaptoethanol (2ME), depending upon experimental requirements. Gels were electrophoresed at a constant voltage of 200 V/gel on a XCell SureLock™ Mini-Cell (Invitrogen, Carlsbad, CA). Resolved proteins were stained with colloidal Coomassie (GelCode Blue Stain Reagent, Pierce, Rockford, IL) according to the manufacturers instructions.

Gels containing larval proteins resolved by SDS-PAGE (with- and without-2ME) were transferred electrophoretically to nitrocellulose with a XCell II™ Blot Module in a NuPAGE® transfer buffer at a constant 30 V for 1 h for Western blotting. The specific details of Western blotting can be found in Pruett et al. (2006). In summary, in this study the blot was blocked with 2% goat's milk and test serum was diluted 1:500 in serum diluent buffer (10 mM sodium phosphate buffer, pH 7.2, containing 1% Tween 80, 0.01% anti-foam A, and 2% goat's milk) and incubated overnight at room temperature with gentle shaking. Antibody binding to BmLF3 proteins was detected with rabbit anti-bovine IgG (whole molecule, heavy + light chains, therefore, not class specific, Sigma Immuno Chemicals, St. Louis, MO) diluted 1:4000, in serum diluent buffer and incubated with blots for 1 h with shaking. Goat anti-rabbit IgG (whole molecule, heavy and light chains, horseradish peroxidase labeled) was used to detect primary antibody binding to BmLF3 proteins at a 1:4000 dilution in serum diluent. Following 1 h of incubation, with shaking, at room temperature the blot was washed (10 mM sodium phosphate buffered saline [0.145 M], pH 7.4, containing 0.3% Tween 20). Reactive proteins bands were visualized with *o*-dianisidine (15 μg/ml) and 0.01% H_2O_2 in 10 mM Tris–HCl, pH 7.5 for 20 min with shaking. Results of SDS-PAGE gels and Western blots were analyzed with the Kodak Gel Logic 440 system and the Kodak Molecular Imaging Software (Eastman Kodak Co., Rochester, NY).

2.6. Trypsin inhibition

The chromagenic substrate L-Bapna (N_α-benzoyl-DL-arginine 4-nitroanilide hydrochloride, Sigma–Aldrich, St. Louis, MO) was solubilized in DMSO and a 1.25 mM working solution was prepared in substrate buffer (0.1 M Tris, pH 8.1, 1 mM $CaCl_2$). Bovine trypsin (Sigma–Aldrich, St. Louis, MO) in 0.001 N HCl was used (2 μg/well) as a positive control for trypsin activity. BmLF3 reverse-phase fractions that were evaluated for trypsin inhibition were rehydrated with 0.1 M Tris, pH 8.1, 1 mM $CaCl_2$.

The standard assay included 10 μl bovine trypsin (2 μg) plus 10 μl inhibitor solution (10 μl, 50 mM sodium phosphate buffer, pH 7.5 for trypsin only control) and 180 μl of substrate (L-Bapna). The reaction was monitored at 405 nm, 30 °C, in an EL808 Ultra Microplate Reader (BioTek Instruments Inc.) with readings at 2 min intervals for 16 min. A buffer control (50 mM sodium phosphate buffer, pH 7.5, no trypsin) was included. Percent activity remaining after 16 min of reaction was determined by comparing the ΔOD_{16min} of the reaction of the potential inhibitor with the uninhibited bovine trypsin reaction.

2.7. Affinity purification of trypsin inhibitory activity

BmLF3 filtrate ≈100 ml from the ultrafiltration of BmLF3 was concentrated to dryness by lyophilization

and reconstituted with 10 ml of 10 mM sodium phosphate buffer, pH 7.2. Concentrated BmLF3 was desalted on a Sephadex G25-40 column with 50 mM ammonium acetate, pH 6.0 buffer. Fraction sizes were 1.7 ml and fractions 6 through 18 were concentrated to dryness with the Savant Speed Vac SC110. Fractions were rehydrated with 0.5 ml of a 50 mM sodium phosphate buffer, pH 7.5. Trypsin inhibitory activity was found in fractions 6 through 11. Those fractions were pooled creating a composite sample that possessed trypsin inhibitory activity. Trypsin binding proteins in the composite sample were affinity purified with a trypsin-agarose column (immobilized TPCK trypsin, Pierce, Rockford, IL). The binding buffer was 50 mM sodium phosphate, pH 7.5 and the elution buffer was 0.2 M potassium chloride in 0.07 M HCl, pH 2.8. The eluted proteins were neutralized with 1 M Tris, pH 8.5, and concentrated to dryness with the Savant Speed Vac SC110. Proteins were rehydrated with distilled water and desalted on the Sephadex G25-40 column. Desalted fractions (1 through 11) were concentrated to dryness with the Savant Speed Vac SC110 and rehydrated with 1.0 ml distilled water per fraction. Trypsin inhibitory activity was found in fractions 3 through 6.

3. Results

3.1. Fractionation of R. microplus soluble proteins

The primary focus of the current report was to characterize the polypeptide constituents of fraction 3 (BmLF3), and as with BmLF2 evaluate the immunogenicity of BmLF3 proteins in the bovine host exposed to those proteins as a result of natural exposure (Fig. 1). Pruett et al. (2006) reported that BmLF3 contained 32 polypeptides (SDS-PAGE, 2ME) ranging in molecular weight from 94.6 to 12.3 kDa, and BmLF2 contained 24 polypeptides ranging in molecular weight from 177 to 12.9 kDa apparently sharing 6 polypeptides of similar mass with BmLF3 (≈43, 27, 17, 14, 13, and 12 kDa; data not shown). In the current study a SDS-PAGE analysis (without-2ME) of these same fractions yielded 20 protein bands for BmLF2, ranging in molecular weight from 271 to 9.2 kDa, and 17 protein bands for BmLF3, ranging in molecular weight from 192.1 to 9.0 kDa. BmLF2 and BmLF3 shared 3 protein bands, at 192.1, 35.2, and 9 kDa (data not shown). Concentration of BmLF2 and BmLF3 to equivalent volumes revealed that BmLF2 contained 11.8 times more protein by weight (4.7 mg/ml vs. 0.4 mg/ml).

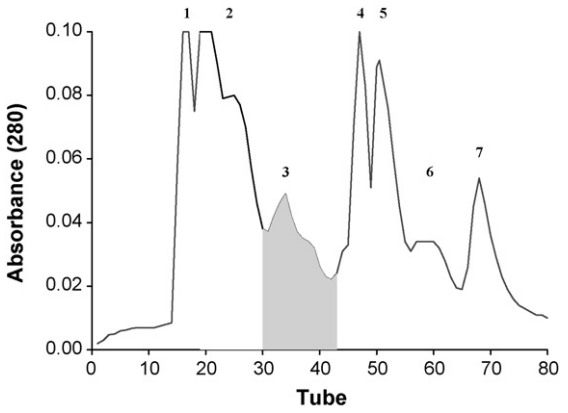

Fig. 1. Sephacryl S-300 molecular-sieve chromatographic separation of soluble crude *R. microplus* larval proteins into seven fractions. Fraction 2 (BmLF2) is a pool of tubes 19–30 evaluated in Pruett et al. (2006), and fraction 3 (BmLF3, shaded area) is a pool of tubes 31–43 and the subject of the current study.

3.2. Western blot analysis of crude BmLF2 and BmLF3 proteins with pre- and post-larval exposure composite sera

Western blot analysis of SDS-PAGE (without-2ME) separated crude BmLF2 and BmLF3 proteins with both a pre-infestation composite pool of antiserum and a post-infestation composite pool of antiserum from the same calves repeatedly infested with *R. microplus* larvae is presented in Fig. 2. Nine major protein antigen bands in BmLF2 were identified by antibodies in the pre-infestation antiserum (229.8, 204.1, 171.9, 123.7, 92.2, 83.6, 59.7, 52.1, and 41.5 kDa). Bands with weak antibody binding were noted at 162.3, 139.8, 107.6, and 21.8 kDa. Analysis of BmLF2 with post-infestation antiserum revealed a loss of the 92.2 kDa band with a gain of 4 antigenic bands (37.6, 33.8, 28.8, and 27.0 kDa). The 21.8 kDa band was identified by the post-infestation antiserum, but was less intense than observed with pre-infestation serum indicative of a drop in concentration. Of the 4 presumptive *R. microplus* specific antigens only bands 33.8 and 27.0 kDa may be specific, as a less stringent Western blot (reduced milk blocking protein, 0.5%) revealed antigenic bands at 37.6, 28.6, 21.2 and 16.8 kDa (data not shown).

Western blot analysis of SDS-PAGE (without-2ME) separated BmLF3 proteins is also presented in Fig. 2. Five major protein antigenic bands were identified by antibody binding in the composite pre-infestation antiserum of experimental calves (171.9, 98.0, 86.5, 58.9 and 38.0 kDa) along with 2 minor antigenic bands (207.3 and 123.7 kDa). Three of these proteins may be shared with BmLF2 by comparison of similar mass

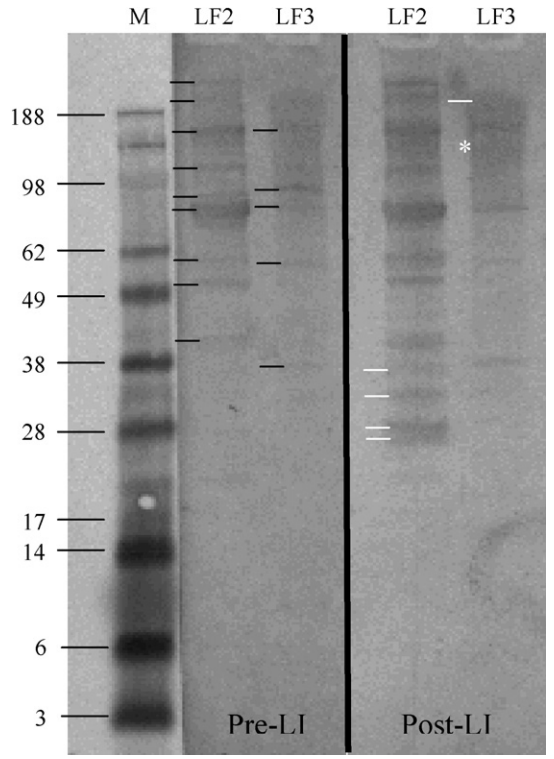

Fig. 2. Western blot analysis of BmLF2 and BmLF3 crude proteins with pooled serum obtained from calves prior to (pre-LI) and post-*R. microplus* larval infestation (post-LI). Black bars represent major antigenic bands identified by pre-LI antiserum, white bars represent major antigenic bands identified by post-LI antiserum, and the white asterisk identifies a minor antigenic band identified in LF3 by post-LI antiserum.

(171.9, 83.6, and 59.7 kDa). Post-infestation composite serum revealed a gain of only 1 major band at 207.3 kDa, which was identified as a minor band by pre-infestation antiserum. A minor band not observed with pre-infestation antiserum was revealed at 149.4 kDa by post-infestation antiserum. The conjugate control revealed very weak binding at bands 140.6, 124.1, and 84.2 kDa for BmLF2; and at 140.3 and 15.7 kDa for BmLF3 (data not shown).

3.3. Reverse-phase HPLC separation of BmLF3 proteins and identification of serologically defined protein antigens with pre- and post-larval exposure composite antisera

BmLF3 proteins were further separated by reversed-phase HPLC into 10 peaks (Fig. 3; peaks 1–10). These peaks were concentrated to dryness and rehydrated and prepared for SDS-PAGE (Fig. 4A, without-2ME). Peaks 6 through 9 yielded the abundant proteins. Protein staining of peak 6 revealed 2 distinct proteins at 21.4 and 13.1 kDa. Western blot analysis of these proteins with composite pre- and post-*R. microplus* larval infestation serum was negative for antibody binding. Peak 7 revealed 7 proteins at 50, 40.5, 31.6, 23.3, 18.2, 10.1, and 8.7 kDa, and as with peak 6 no antigenic proteins were detected by Western blotting with pre and post-infestation composite antiserum. Peak 8 had the greatest number of protein bands with 19 at 140.2, 126.4, 119.6, 100, 89.5, 67.4, 55.1, 52.2, 48.4, 45.2, 38.5, 30.2, 22.9, 17.8, 15.5, 12.4, 9.8, 7.2, and 5.8 kDa. Pre-*R. microplus* infestation composite antiserum revealed 4 presumptive ixodid common antigens at 95.8, 85.5, 64.9 and 37.3 kDa in peak 8 (Fig. 4B), while post-infestation composite antiserum revealed similar antigenic bands at 95.8, 84.8, 65.7, and 36.7 kDa, plus an apparent *R. microplus* specific band at 116.0 kDa. Peak 9 had 8 protein bands at 236.1, 139.2, 127.4, 93.8, 70, 54.5, 49, and 29.3 kDa. Pre-*R. microplus* composite infestation antiserum revealed 3 presumptive ixodid common antigens at 94.3, 87.0, and 59.6 kDa, while post-infestation composite antiserum revealed similar antigenic bands at 95.8 and 85.5 kDa, plus a band at 65.7 kDa that was identified by pre-infestation composite antiserum in peak 8 above. The conjugate control was negative for binding to any BmLF3 protein.

3.4. Inhibition of trypsin activity by BmLF3

Larval Sephacryl S-300 fractions 1 through 4 (BmLF1–BmLF4, Fig. 1) were evaluated for their ability to inhibit trypsin hydrolytic activity. BmLF3 (4.0 μg in assay) was found to inhibit trypsin at 92.4%

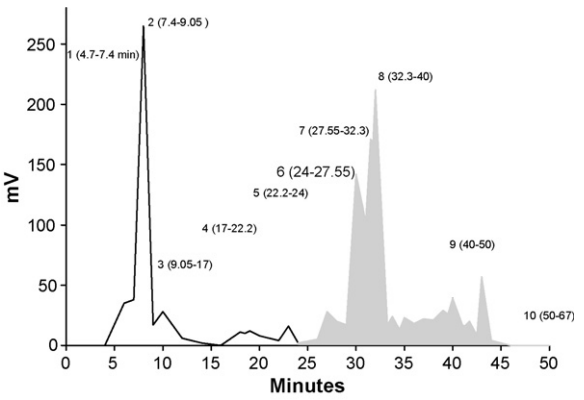

Fig. 3. Reverse-phase HPLC separation of BmLF3 proteins into 10 peaks with a linear gradient of 0.1% TFA to 100% acetonitrile containing 0.1% TFA over 67 min. Peaks 6 through 9 were found to contain the abundant larval proteins, with peaks 7 and 8 containing trypsin inhibitors. Numbers in parentheses represent elution times in minutes.

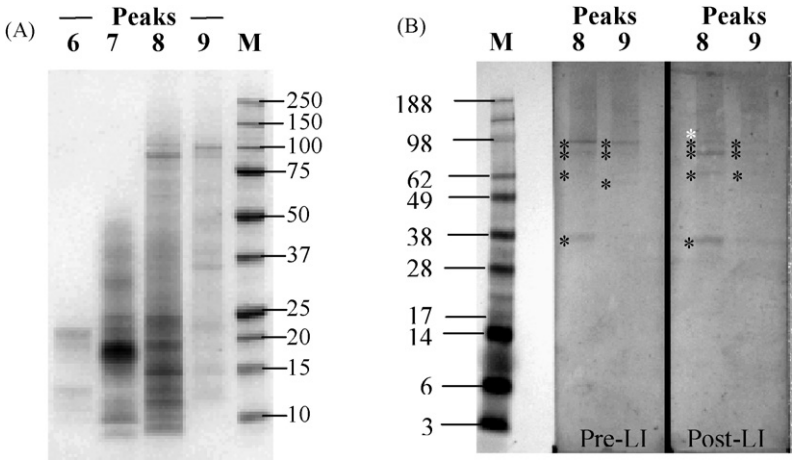

Fig. 4. (A) SDS-PAGE (without-2ME) analysis of BmLF3 proteins fractionated by reverse-phase HPLC. Peaks 6 through 9 were resolved on a gradient (4–12%) polyacrylamide gel. M denotes molecular weight markers. (B) Western blot analysis of BmLF3 reverse-phase HPLC peaks 8 and 9 with pooled serum obtained from calves prior to (pre-LI) and post-*R. microplus* larval infestation (post-LI). Proteins were resolved as in (A). Black stars indicate potential ixodid common antigens and the white star identifies a potential *R. microplus* specific antigen (116.0 kDa).

inhibition relative to the uninhibited control reaction, followed by BmLF2 (46.95 μg), 12.2%; BmLF4 (0.19 μg), 4.5%; and BmLF1 (5.83 μg), 1.5%. A further evaluation of reverse-phase fractions of BmLF3 (Fig. 3) revealed that trypsin inhibitory activity was concentrated in peak 7, 91.8% inhibition, and peak 8, 99.4% inhibition. Peak 6 (3.8% inhibition) and peak 9 (2.4%) proteins were essentially negative for inhibitory activity. In addition, the filtrate from the concentration of BmLF3 (1.85 μg) without further concentration inhibited trypsin hydrolytic activity at 43.1%.

3.5. Trypsin-agarose affinity chromatography purification of BmLF3 filtrate trypsin inhibitors

Concentrated BmLF3 filtrate was desalted on a Sephadex G25-40 column and tubes containing trypsin inhibitory activity (6–11) were pooled. The pooled concentrate was applied to a trypsin-agarose affinity column and bound proteins eluted into 4 fractions (fraction 3–6) that inhibited trypsin hydrolytic activity. Analysis of the eluted fractions with SDS-PAGE (with-2ME) yielded polypeptides of 16.6, 13.3, and 10.5 kDa in each fraction (Fig. 5A). Analysis of these proteins by

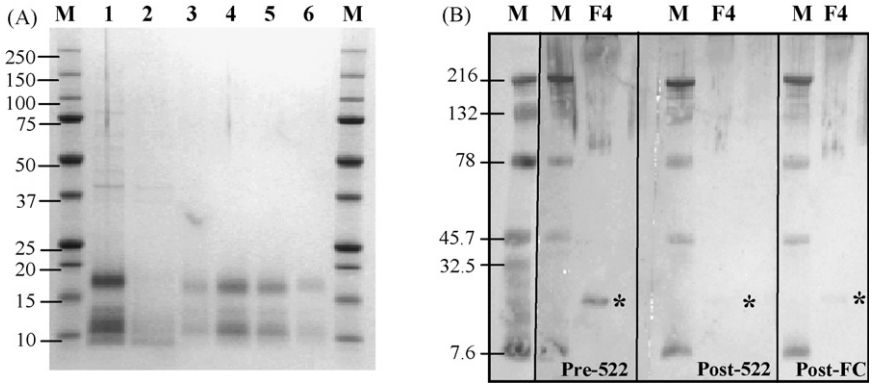

Fig. 5. (A) SDS-PAGE (with-2ME) analysis of BmLF3 filtrate proteins affinity purified on a trypsin-agarose affinity column with a gradient (4–12%) polyacrylamide gel. M denotes molecular weight markers. Lane 1, crude desalted BmLF3 filtrate proteins (pooled tubes 6–11); lane 2, fall through BmF3 filtrate proteins (not affinity bound); lanes 3 through 6, affinity bound proteins fractionated on a desalting column. (B) Western blot analysis of affinity pure fraction 4 (F4 from A, lane 4) with calf 522 serum prior to infestation with *R. microplus* larvae (pre-522), 522 serum collected 21 days following the 5th infestation with larvae (post-522), and a composite serum sample of 4 calves bled 28 days following the 3rd infestation of the full life-cycle of *R. microplus* (larvae, nymphs, adults, post-FC). The (*) denotes antibody binding to the 16.6 kDa protein.

Western blot with antiserum from a pre- and post-*R. microplus* larval infested calf (522) and post-infestation pooled composite antiserum from calves infested with all life-stages (complete life-cycle) yielded weak antibody binding to the 16.6 kDa protein with the serum from calves infested with all life-stages, however, antibody binding to the 16.6 kDa in control pre-infestation serum was noted as well (Fig. 5B). The conjugate control blot was negative.

4. Discussion

Cattle acquire a protective immunological response to tick infestation as a result of repeated natural exposure (Wikel and Whelan, 1986). The phenotype of this protective response is primarily manifested as an inability to obtain an adequate blood meal by all life-stages (Wikel, 1988; Panda et al., 1993). The protective tick antigens, presumably exposed to the host via salivary secretions (Ribeiro and Francischetti, 2003), are poorly known.

The identification of larval immunogenic proteins in S-300 Sephacryl molecular-sieve chromatography fraction 3 (BmLF3) was the primary subject of this study. In a previous study (Pruett et al., 2006), the immunogenic proteins in BmLF2 were investigated using an antiserum from the calf in our collection with the highest concentration of BmLF2 antibodies. In the current study, we have utilized pre-larval infestation and post-larval infestation pools of antiserum for Western blot analysis of crude BmLF2 and BmLF3, and HPLC fractionated BmLF3. The use of pooled antiserum most likely reveals the common antigens recognized by different calves, as individual differences in response to specific immunogenic proteins may be masked by the dilution of an individual's serum (1/4 × working dilution).

In the current study, antibodies in pre-infestation pooled serum bound 12 major protein bands, 9 in BmLF2 of which 3 where shared in BmLF3 (171.9, 83.6, and 59.7 kDa), 2 unique in BmLF3 (98.0 and 38.0 kDa) and 1 unique protein shared between BmLF3 HPLC peaks 8 and 9 (65.7 kDa). *R. microplus* was eradicated from the U.S. in 1943 and a quarantine zone, and an eradication program along the Rio Grande River has been maintained since to prevent reintroduction of the tick (Graham and Hourrigan, 1977). However, we found considerable anti-*R. microplus* antibody activity in the serum of calves having a very low probability of *R. microplus* exposure. The number of *R. microplus* protein bands identified by pre-infestation serum was quite unexpected. We reason that these antibodies were most likely elicited to shared proteins of other ixodid ticks (e.g. *Amblyoma americanum*) as both these calves and their dams could have been exposed to other ixodids on pasture prior to our purchase of the calves. Although, these antibodies could have been elicited to cross-reactive epitopes shared by other arthropods, or parasites, the shared ixodid protein argument is strengthened by the observation that antibodies elicited by *R. microplus* exposure, evidenced by increased signal intensity (darker band), bound many of the same bands as those antibodies found in the pre-infestation pooled serum. In fact, only 3 additional major *R. microplus* specific bands were identified by antibodies in the post-infestation pool antiserum, as 2 in BmLF2 (33.8 and 27.0 kDa) and 1 in BmLF3 HPLC fractionated peak 8 (116 kDa) were observed. In general, the bovine humoral response to larval proteins appears weak. This was demonstrated by the faintness of the antibody binding signal intensity with Western blots, thus indicating a low concentration of antibody in response to larval natural exposure.

Larval fraction BmLF3 and BmLF3 filtrate were found to possess significant trypsin inhibitory activity relative to the other BmLF S-300 Sephacryl fractions. Larvae of *R. microplus* are known to be an abundant source of bovine pancreatic trypsin inhibitor (BPTI)–Kunitz type serine proteinase inhibitors (Sasaki et al., 2004). These trypsin inhibitors (BmTI's) are quite numerous and range in molecular weight between 18.4 and 6.2 kDa. Several BmTI's share the same molecular mass but bind differently to ion-exchange columns. For example, Sasaki et al. (2004), beginning with BmTI's isolated by trypsin-sepharose affinity chromatography, identified 6 different peaks by anion chromatography and 6 different peaks by cation chromatography for a total of 12 different peaks representative of individual BmTI's. In an earlier report (Tanaka et al., 1999), these researchers described a double-headed serine proteinase inhibitor from *R. microplus* larvae. Two protein bands of 18 and 10 kDa were identified that inhibited trypsin (Tanaka et al., 1999). Only the 18 kDa protein fraction inhibited human plasma kallikrein and neutrophil elastase, as well as increased the activated partial thromboplastin time (Tanaka et al., 1999). The biological significance of these findings suggest that in addition to the roles these inhibitors may play in normal tick physiology, they may also aid in the attainment of a host blood meal as they enter the feeding site via salivary gland secretions (Tanaka et al., 1999). This mediator, by inhibiting kallikrein, can potentially increase coagulation time and avoid the accumulation

of edema by inhibition of local bradykinin release (Ribeiro, 1989).

Sasaki et al. (2004) have demonstrated that vaccination of cattle with unfractionated BmTI's can elicit antibodies to several of the purified BmTI's. These unfractionated BmTI's have been used as vaccine antigens in a challenge trial study and found to be host protective in terms of 72% efficacy in the interference with tick development and a 69.7% reduction in tick number, and a 71.3% reduction in egg weight (Andreotti et al., 2002).

In view of larval BmTI's characterized previously (Tanaka et al., 1999), it appears that BmLF3 contains BmTI's based upon trypsin inhibitory activity, binding to a trypsin-agarose affinity column, and similar molecular mass. However, in contrast with the vaccination results of Andreotti et al. (2002) it does not appear that a significant humoral immune response to the BmTI's occurs in the host as a result of natural tick exposure. Apparent antibody binding to the 16.6 kDa protein by antibodies in pre-infestation serum suggests, as previously discussed, cross reaction with homologous antigens that are shared with other ixodid species. As compared to *A. americanum*, *R. microplus* is presumed to be fairly host-specific and a one host tick. In our experimental infestations of both larval specific, and the complete life-cycle (larvae, nymph, and adult) an increase in the concentration of anti-16.6 kDa antibody was not observed as a result of natural exposure. Although some antibody binding could be observed in the post larval and post full life-cycle sera, those antibodies that bound cannot be distinguished from a potential declining concentration in the pre-infestation sera. If one assumes that these BmTI's enter the host via saliva, then it appears that either the bovine host is unresponsive to them as a result of natural exposure or if antibodies are elicited they are in very low concentration or potentially tissue fixed. Future studies should include evaluation of purified BmTi's in skin test assays for tissue fixed antibodies.

In summary, several *R. microplus* larval antigenic proteins are recognized by antibodies in pre- and post-*R. microplus* infestation pooled antiserum. However, even with repeated natural exposure the humoral response to larval proteins is minimal as evidenced by the low concentration of antibody as defined by the faintness of signal intensity with the Western blot results. This observation would suggest that the minimal response was due to the immunogenicity of these proteins or the dose of immunogen presented to the host by natural larval exposure. This research question may be answered with purification of the immunogenic proteins followed by dose-response studies with pure protein.

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